In contrast, the use of the mobility shift assay to obtain titration curves that separately represent each protein-DNA complex with exactly n bound proteins requires that the protein-DNA complexes do not dissociate in the gel during electrophoresis and that their distribution remains unchanged. It has been noted that quantitation of the unbound DNA band is less affected by dissociation of protein-DNA complexes in the gel. If only the unbound DNA is quantitated, however, all of the information concerning potential cooperative free energies is lost. For a theoretical discussion of the determination of cooperativity constants from the mobility shift assay, see Senear et al. (1986).

UV Crosslinking of Proteins to Nucleic Acids

Irradiation of protein-nucleic acid complexes with ultraviolet light causes covalent bonds to form between the nucleic acid and proteins that are in close contact with the nucleic acid. Thus, UV crosslinking may be used to selectively label DNA-binding proteins based on their specific interaction with a DNA recognition site. As a consequence of label transfer, the molecular weight of a DNA-binding protein in a crude mixture can be rapidly and reliably determined.

The procedure can be divided into 3 stages: (1) Extract containing the protein of interest is incubated with a radioactive, uniformly labeled DNA fragment that contains a high-affinity binding site for the protein; (2) protein-DNA complexes are crosslinked with UV irradiation and digested with nuclease, leaving only those labeled DNA fragments that are crosslinked and in close contact with the DNA-binding protein; and (3) the molecular weights of the crosslinked proteins are determined by SDS-polyacrylamide gel electrophoresis followed by autoradiography. One of the advantages of this technique is that proteins bound specifically to the DNA probe can be easily distinguished from those bound nonspecifically.

BASIC PROTOCOL: UV CROSSLINKING USING A BROMODEOXYURIDINE-SUBSTITUTED PROBE

DNA molecules containing halogenated analogs of thymidine, such as bromodeoxyuridine (BrdU), are considerably more sensitive to UV-induced crosslinking compared to unsubstituted DNA. Although use of BrdU-substituted probes is not essential for detecting protein-DNA crosslinking by UV light, in many cases it is helpful.

COMMENTARY

Crosslinking proteins to nucleic acids with UV light is a simple method for rapidly and accurately determining the molecular weight of a DNA-binding protein in a crude extract. Moreover, the specificity of the photoadduct can be rigorously determined by measuring the ability of an excess of unlabeled competitor DNA to compete for binding sites on the protein.

The goal of the UV crosslinking method is to specifically transfer a radioactive label from a

DNA-binding site to the binding protein. Irradiation of DNA with UV light produces purine and pyrimidine free radicals. If a protein molecule is in close proximity to the free radical, a covalent bond can be formed, crosslinking the protein to the DNA.

In solution, pyrimidines are approximately 10-fold more sensitive to photochemical alteration than purines. Several amino acids are known to form photoadducts with pyrimidine bases, including cysteine, serine, methionine, lysine, arginine, histidine, trytophan, phenylalanine, and tyrosine. Cells that have incorporated halogenated analogs of thymine--such as bromodeoxyuridine (BrdU)--into their DNA are several times more sensitive to UV-induced crosslinking with protein than normal cells. This is because replacement of the thymidine methyl group with the bromine atom creates a molecule more susceptible to free radical formation in the presence of UV light. Because the bromo group is approximately the same van der Waals radius as a methyl group, several cellular enzymes will use thymidine and BrdU interchangeably. Thus, it is quite simple to generate BrdU-substituted DNA probes.

Another important reason for using BrdU is that the longer wavelength of UV light used to crosslink these probes is less damaging to proteins than a shorter wavelength. In addition, substitution of BrdU into a binding site sometimes increases the affinity of the protein-DNA interaction being studied.

Purification of DNA-Binding Proteins Using Biotin/Streptavidin Affinity Systems

Short fragments of DNA--either natural or formed from oligonucleotides--containing a high-affinity site for a DNA-binding protein provide a powerful tool for purification. The biotin/streptavidin purification system is based on the tight and essentially irreversible complex that biotin forms with streptavidin. The experimental design of this system is illustrated in Figure 12.6.1. First, a DNA fragment is prepared that contains a high-affinity binding site for the protein of interest. A molecule of biotinylated nucleotide is incorporated into one of the ends of the DNA fragment. The protein of interest is allowed to bind to the high-affinity recognition site present in the biotinylated fragment. The tetrameric protein streptavidin is then bound to the biotinylated end of the DNA fragment. Next, the protein/biotinylated fragment/streptavidin ternary complex is efficiently removed by adsorption onto a biotin-containing resin. Since streptavidin is multivalent, it is able to serve as a bridge between the biotinylated DNA fragment and the biotin-containing resin. Proteins remaining in the supernatant are washed away under conditions that maximize the stability of the DNA-protein complex. Finally, the protein of interest is eluted from the resin with a high-salt buffer.

ALTERNATE PROTOCOL: PURIFICATION USING A MICROCOLUMN

Although the batch method in the basic protocol is rapid and well-suited for analytical-scale purification, larger volumes of biotin-cellulose resin can be better handled in a microcolumn. This method is also used to elute the protein in as small a volume (i.e., as high a concentration) as possible.

ALTERNATE PROTOCOL: PURIFICATION USING STREPTAVIDIN-AGAROSE

When high-quality free streptavidin is not available or cellulose is an inappropriate resin, a simple